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Liprotides assist in folding of outer membrane proteins

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Abstract: Proteins and lipids can form complexes called liprotides, in which the partially denatured protein forms a shell encasing a lipid core. This effectively stabilizes a lipid micelle in an aqueous solvent and suggests that liprotides may provide a suitable vessel for membrane proteins. Accordingly we have investigated if liprotides consisting of α-lactalbumin and oleate could aid folding of four different outer membrane proteins (OMPs) tOmpA, PagP, BamA, and OmpF. tOmpA was able to fold in the presence of the liprotide, and folding did not occur if only oleate or α-lactalbumin were added. Although the liprotides did not fold the other three OMPs on its own, it was able to assist their folding in the presence of vesicles. Incubation with liprotides before folding into vesicles increased the folding yield of the outer membrane proteins to a level higher than using micelles of the non-ionic surfactant DDM. Even though the liprotide was stable at both high urea concentrations and high pH, it failed to efficiently fold OmpA at high pH. Instead, optimal folding was seen at pH 8–9, suggesting that important changes in the liprotide occurred when increasing the pH. We conclude that an otherwise folding-inactive fatty acid can be activated when presented by a liprotide and thereby work as an *in vitro* chaperone for outer membrane proteins.

Keywords: liprotide; outer membrane proteins; folding; chaperone; tOmpA; PagP; BamA; OmpF

Introduction

Gram-negative bacteria contain both an inner and an outer membrane. The outer membrane contains lipids such as lipopolysaccharides not found in the inner membrane, and is highly asymmetric with respect to its inner and outer leaflets. Furthermore, it is home to the outer membrane proteins (OMPs). OMPs have a diverse set of functions including

Abbreviations: aLA, α-lactalbumin; apo-aLA, Ca^{2+} depleted α-lactalbumin; CD, Circular dichroism; CMC, critical micelle concentration; DDM, n-Dodecyl-β-D-Maltopyranoside; DLPC, 1,2-dilauroyl-sn-Glycero-3-phosphocholine; IBs, inclusion bodies; liprotides, complexes between lipids and partially denatured proteins; OA, oleate; OMP, outer membrane protein; *p*(*r*), pair distance distribution function; SAXS, small-angle X-ray scattering; SUV, small unilamellar vesicles.

Additional Supporting Information may be found in the online version of this article.

Description of supplementary figures: Fig. S1: Fit of SAXS data at different [urea] Fig. S2: SDS-PAGE of folding of PagP, tBamA, and OmpF.

Statement: Bacterial outer membrane proteins (OMPs) need a hydrophobic environment to fold and be functional. We used a number of biophysical techniques to investigate if so-called liprotides, in which a protein shell encases a lipid core, could assist folding of four different OMPs. One OMP was folded entirely by liprotides while liprotides helped transfer the three others to the membrane, suggesting a useful role for liprotides in OMP refolding.

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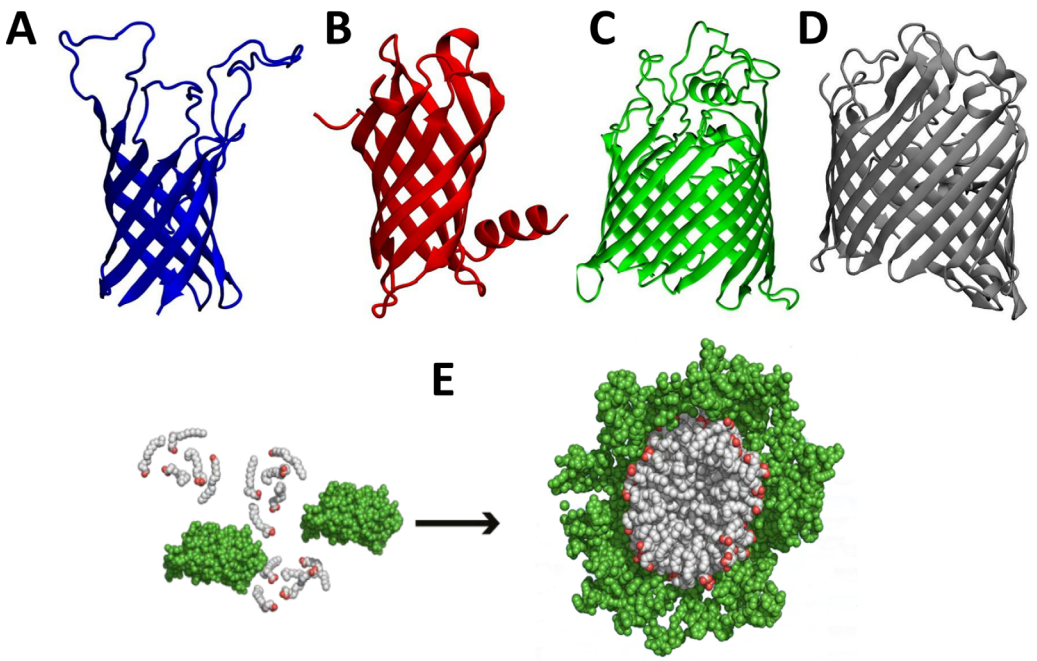

Figure 1. Crystal structure of outer membrane proteins and formation of liprotide. (A) tOmpA[41] (18.7 kDa, pdb:1bxw). (B) PagP[42] (19.1 kDa, pdb:1thq). (C) tBamA[43] (43.2 kDa, pdb:4n75). (D) OmpF[44] (37.1 kDa, pdb:2zfg). E) α-lactalbumin (green) and oleate (white) go together to form a core-shell liprotide (adopted from Kaspersen *et al*.[12]). The structures in A–D were visualized using VMD.[45]

enzymes, folding catalyst for other OMPs, structural proteins, diffusion pores and efflux channels[1] but all consist of β-barrels formed by an even number of β-strands (most commonly 8–16 strands, but up to 26 have been reported).[2] Because of their β-sheet structure, OMPs typically consist of alternating hydrophobic and hydrophilic residues. This reduces their overall hydrophobicity compared to α-helical membrane proteins and makes it easier to refold them from their urea denatured state.[3] The asymmetry of the lipid composition in the outer membrane makes *in vitro* manufacturing of realistic vesicles difficult and most often simple phospholipid vesicles or detergent micelles are used for folding. *In vivo*, several different chaperones are involved in proper stabilization and folding of OMPs into the membrane.[4]

tOmpA [Fig. 1(A)] is an intensely studied 8-strand OMP known to refold *in vitro* in a wide range of vesicles and detergents.[5–7] Other OMPs such as the 8-stranded PagP and the 16-stranded OmpF and BamA [Fig. 1(B–D)] also fold in a wide range of detergents, but folding yields are generally lower than for tOmpA.[8,9] Folding of OMPs is generally improved by increasing lipid bilayer curvature (using small vesicles)[10] and decreasing bilayer thickness (using short chained lipids).[7,11] However, *in vitro* folding of OMPs is generally challenging. For this reason we looked for a way to assist this folding using new ways of presenting lipids to the OMPs.

One such possibility is provided by a class of complexes which we term "liprotide" [Fig. 1(E)] to describe complexes between lipids and partially denatured proteins[12]. These complexes, originally discovered in milk by Svanborg and co-workers,[13] consist of a micelle core of oleate (OA) with a partially denatured protein shell. The protein α-lactalbumin (aLA) is particularly good at forming liprotides. Circular dichroism (CD) and Trp fluorescence have shown that aLA upon liprotide formation undergoes large tertiary structural changes in conjunction with smaller changes in secondary structure.[14,15] Liprotides kill cancer cells but not healthy differentiated cells,[13] have antibacterial properties[16] and can stabilize and solubilize small hydrophobic compounds.[17,18] OA is generally not soluble at neutral pH but the protein shell helps to solubilize the lipid component.[19] In turn, the lipid component is responsible for solubilization of small compounds as well as effects on cancer cell and bacteria.[19] Several studies have shown that many different proteins[12,20,21] and *cis*-unsaturated fatty acids[22,23] form liprotides, suggesting that this is a general feature of the polypeptide chain when combined with flexible lipid chains. We chose to use liprotides consisting of aLA and OA which are easily available, intensely studied and form spontaneously at room temperature.

Here we investigate if liprotides can assist in folding of transmembrane tOmpA, PagP, transmembrane BamA (tBamA), and OmpF. We observe that liprotides persist at molar concentrations of the denaturant urea without markedly changing their structure, making them useful for refolding under

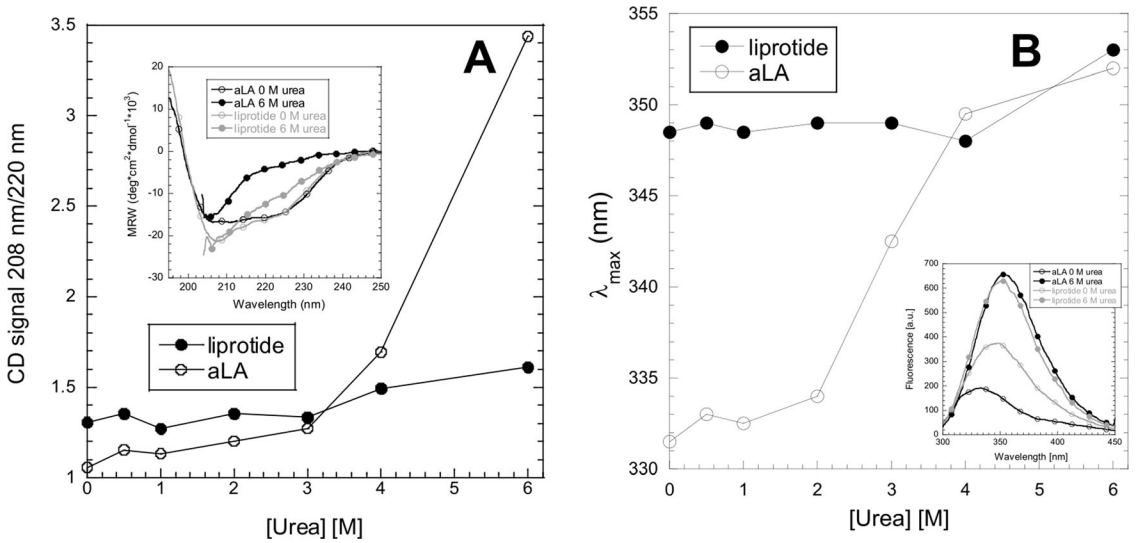

Figure 2. Structural changes in α-lactalbumin and liprotide measured with CD and Trp fluorescence. (A) Intensity at 208 nm divided by 220 nm of a CD measurement as a function of [urea]. Insert shows CD spectrum of aLA and liprotide with lowest (0 M) and highest (6 M) [urea]. (B) Max intensity (λ_{max}) of Trp fluorescence measurement as a function of [urea]. Insert shows Trp fluorescence of aLA and liprotide with lowest (0 M) and highest (6 M) [urea].

conditions that reduce protein aggregation. Further, liprotides but not OA alone are able to fold tOmpA. Folding is very pH dependent and only occurs completely for tOmpA. For PagP, OmpF, and tBamA the liprotide can assist in the folding of OMPs into micelles most likely by providing a micelle-like environment similar to non-ionic detergents like DDM. This dramatically expands the size range for liprotide substrates, extending liprotide applications and contributing to our understanding of OMP folding.

Results

Liprotides are stable up to 4–6 M urea

Small amounts of urea are present when folding OMPs; urea is also sometimes used to facilitate folding of OMPs.[24] We therefore investigated if liprotides were stable towards urea. Liprotides were formed at 0 M urea and 0–6 M urea was added. Circular dichroism (CD), Trp fluorescence and Small-Angle X-ray Scattering (SAXS) were used to monitor any structural changes that might occur when changing [urea]. In 0 M urea, aLA shows a far-UV CD spectrum typical for α-helical structure which is only slightly altered upon liprotide formation [Fig. 2(A)]. In 6 M urea, free aLA assumes a random coil spectrum. In contrast, the liprotide spectrum still has some α-helical structure signal and could be satisfactorily reconstructed by combining 28% of the spectrum of aLA unfolded in 6 M urea with 72% of the spectrum of liprotide in 0 M urea (data not shown), indicating that it is much closer to the original liprotide structure than to a random coil. To follow changes with increasing [urea], we plotted the ellipticity ratio $\theta_{208nm}/\theta_{220nm}$ for all [urea] [Fig. 2(A)]. Higher ratios are indicative of isolated helices and/or random coil.[25] This makes it clear that free aLA undergoes unfolding from 3 to 6 M urea, while aLA in the liprotide retains most of its structure, though small changes are seen around 3–6 M urea. Trp fluorescence, here used to monitor tertiary structural changes, reveals a clear redshift from folded apo-aLA to aLA in liprotide, suggesting that the Trp residues in the liprotide are much more solvent exposed [Fig. 2(B)]. When 6 M urea is added, the liprotide signal is slightly redshifted while the signal for apo-aLA is highly redshifted to a value similar to that of the liprotide. To follow the change at different [urea], we have taken the wavelength at the maximum intensity and plotted as a function of [urea] [Fig. 2(B)]. For apo-aLA, changes start occurring around 2–3 M urea, while the small changes seen for the liprotide occur around 4–6 M urea.

The small changes in CD and Trp fluorescence of the liprotides in 6 M urea could arise both due to structural changes of aLA in the liprotide shell or because the liprotide simply dissociates, leaving some aLA free in solution. To resolve this, we turned to SAXS which reports on the overall structure of the liprotide at different [urea]. The resulting SAXS measurements showed a decrease in intensity extrapolated to a scattering vector q value of 0 ($I(0)$) while the bump at higher values of q was retained as [urea] increased [Fig. 3(A)]. A decrease in $I(0)$ is expected, since urea has a higher electron density than water and the water-urea background level therefore approaches an electron density closer to that of protein as [urea] increases. To compare the overall structure at different [urea], we inspect the samples' $p(r)$ functions, which depict the distribution of scattering distances in the sample [Fig. 3(B),

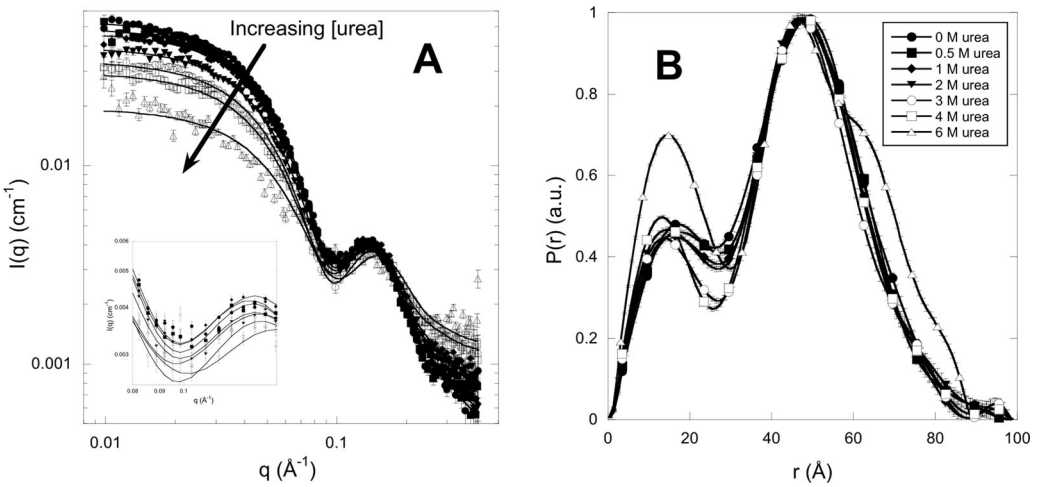

Figure 3. Structural changes in α-lactalbumin and liprotide measured with SAXS. (A) SAXS spectrum of liprotide containing different amounts of urea. The direction of the arrow indicates increasing [urea]. Inset shows zoom around $q = 0.1$ Å^{-1}. (B) The pair distance distribution function $p(r)$ of liprotides determined from measurements in panel A.

intensities normalized for comparison]. These functions show that the overall sizes of the structures in solution are similar for all [urea], in all cases leading to a maximum size D_{max} around 9.0–9.5 nm. Note that the local minimum around 25 Å becomes more pronounced as [urea] increases. We attribute this to the increasing contrast of OA and decreasing contrast of aLA as the solvent electron density increases with urea. We next fitted the liprotide model to the SAXS data using different electron densities for the solvent, calculated for the different [urea]. It was generally possible to get reasonable fits for all [urea] (Fig. 3), when only the shell thickness (D_{shell}), the core radius (R_{In}) and the number of random coils (N_{RC}) were allowed to vary (Table I). Individual fits are shown in Figure S1. Samples containing 0–4 M urea all had χ^2 values < 1.4 and had a core radius and shell thickness within 1 Å of each other, suggesting that urea hardly made a difference to the structure of the liprotides in this concentration range. Size exclusion chromatography data shows that the liprotides mainly elutes as one peak (data not shown) and together with the SAXS data it suggests that the samples containing 0–4 M urea are generally monodisperse. At 6 M urea, the fit became slightly worse ($\chi^2 = 2.34$) and the core radius, the shell thickness and the number of random coils decreased, suggesting that the structure either changed or that part of the liprotide had been destroyed, releasing free aLA and OA in solution. However, we conclude that liprotides are not compromised in any way in 0–1 M [urea] used in our OMP refolding experiments.

Liprotides are stable at pH 7.4–12

OmpA is known to fold at high pH.[26] We therefore needed to establish whether liprotides maintain their structure at higher pH. We first used CD and Trp fluorescence to elucidate structural changes at the protein level accompanying an increase in pH from pH 7.4 to 12 (Fig. 4). Between pH 9 and 11, free aLA responded with an increase in $\theta_{208nm}/\theta_{220nm}$ and a red-shift in emission fluorescence, in both cases to values very similar to those of the

Table I. *Parameters Used for Core-Shell Fit of SAXS Data with Varying [Urea]*

Urea [M]	Electron density (e/Å^3)[a]	χ^2[b]	D_{shell} (Å)[c]	R_{In} (Å)[d]	N_{RC}[e]	OA/aLA[f]	aLA[g]
0	0.334	1.14	24.4 ± 0.2	14.1 ± 0.1	11.3 ± 0.2	8	3.0
0.5	0.336	1.39	24.0 ± 0.2	14.0 ± 0.1	11.4 ± 0.3	8	3.0
1	0.339	1.36	23.7 ± 0.2	14.0 ± 0.1	11.1 ± 0.3	8	3.0
2	0.344	1.30	24.0 ± 0.2	13.4 ± 0.1	10.2 ± 0.3	7	3.0
3	0.349	1.15	23.4 ± 0.2	13.4 ± 0.1	11.2 ± 0.4	8	3.0
4	0.354	1.10	23.8 ± 0.2	13.0 ± 0.1	9.8 ± 0.4	7	3.1
6	0.364	2.34	21.9 ± 0.2	12.2 ± 0.2	7.5 ± 0.8	6	2.2

[a] Electron density of background.
[b] Measure of the quality of the fit to data.
[c] Thickness of shell.
[d] Core radius.
[e] A measure of disorder in the shell.
[f] Number of OA molecules per protein in the complexes.
[g] Number of aLA per complex.

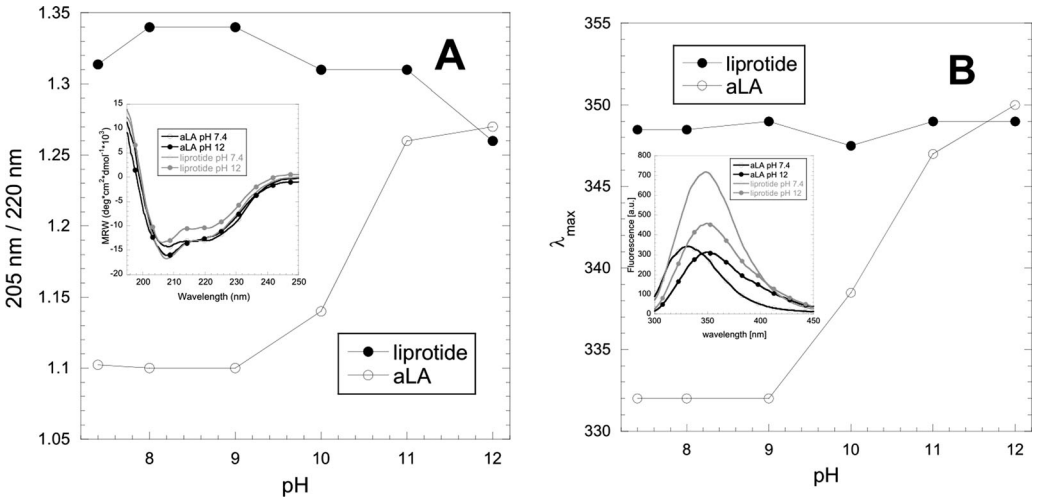

Figure 4. Structural changes in α-lactalbumin and liprotide measured with CD and Trp fluorescence. (A) Intensity at 208 nm divided by 220 nm of a CD measurement as a function of pH. Insert shows CD spectrum of aLA and liprotide with the lowest pH of 7.4 and highest of 12. (B) Max intensity (λ_{max}) of Trp fluorescence measurement as a function of pH. Insert shows Trp fluorescence of aLA and liprotide with the lowest pH of 7.4 and highest of 12.

liprotide. Over this whole pH range, the signal from the liprotide was essentially constant (Fig. 4). This indicates that folded aLA becomes more solvent-exposed and unfolded at higher pH, in agreement with previous studies.[27] In contrast, aLA in the liprotide is already unfolded and no shift in the peak was seen when the pH was changed. There was however a significant decrease in liprotide signal intensity which could be caused by changes in the environment surrounding the Trp or overall changes in liprotide structure. To address this, we recorded SAXS spectra between pH 7 and 12 (Fig. 5). The local minimum around 0.1 $Å^{-1}$ clearly becomes more pronounced at higher pH, showing an increase in symmetry of the complex. The $p(r)$ functions reveal a similar overall core-shell structure at all pH values with a local minimum around 20 Å and maximal dimensions around 90 Å. When we fitted a core-shell model to the data (Fig. 5 and Table II), the most characteristic changes were a decrease in the shell thickness (D_{shell}) and an increase in the number of random coils (N_{RC}). As scattering from the random coils scales as $1/N_{RC}$ relative to the forward scattering I(0), this means that the influence of this fluctuation term is reduced, which together with the decrease in shell thickness shows that there is a compaction of the protein shell. This suggests that the packing of the protein around the micelle changes. The aggregation state of the liprotide, judging from $I(0)$ determined from the $p(r)$ functions

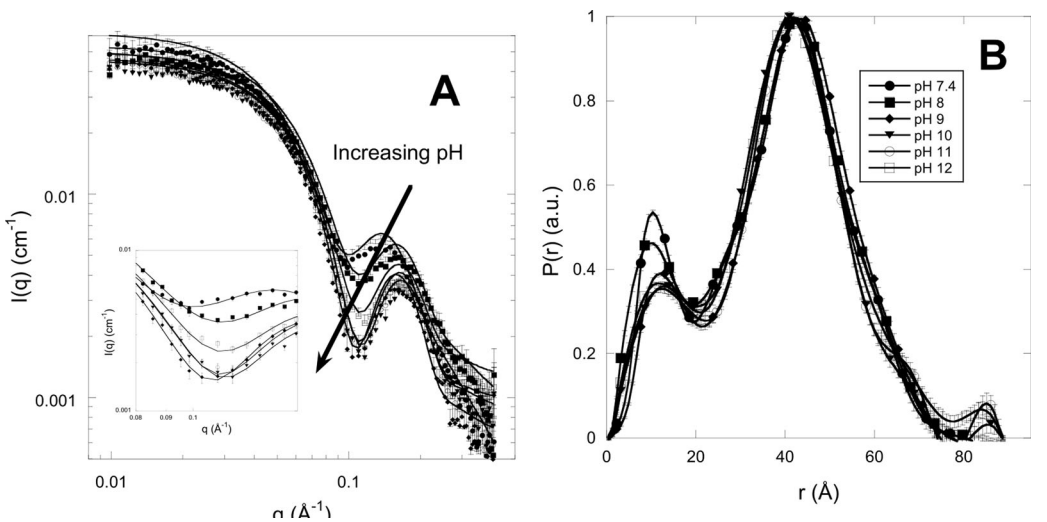

Figure 5. Structural changes in α-lactalbumin and liprotide measured with SAXS. (A) SAXS spectrum of liprotide at different pH. The direction of the arrow indicates increasing pH. Inset shows zoom around $q = 0.1$ $Å^{-1}$. (B) The pair distance distribution function $p(r)$ of liprotides determined from measurements in A.

Table II. *Parameters Used for Core-Shell Fit of SAXS Data with Varying pH*

pH	χ^{2a}	D_{shell} (Å)[b]	R_{In} (Å)[c]	N_{RC}[d]	OA/aLA[e]	aLA[f]	I(0)[g]
7.4	1.85	22.2 ± 0.2	15.2 ± 0.1	8.4 ± 0.8	9	3.2	0.0571
8.0	2.41	18.0 ± 0.3	14.7 ± 0.1	10.8 ± 0.4	10	2.8	0.0492
9.0	2.19	18.0 ± 0.3	15.1 ± 0.1	28.8 ± 2.6	10	2.7	0.0464
10.0	1.83	17.3 ± 0.3	14.4 ± 0.1	33.1 ± 3.5	10	2.4	0.0429
11.0	2.30	17.0 ± 0.3	14.9 ± 0.1	28.2 ± 2.8	10	2.5	0.0439
12.0	3.30	18.0 ± 0.3	14.3 ± 0.2	22.3 ± 2.0	10	2.6	0.0473

[a] Measure of the quality of the fit to data.
[b] Thickness of shell.
[c] Core radius.
[d] A measure of disorder in the shell.
[e] Number of OA molecules per protein in the complexes.
[f] Number of aLA per complex.
[g] Estimated intensity at $q = 0$.

(Table II) also decrease slightly until pH 10, after which it starts to increase again as is also seen by the estimated number of proteins per liprotide (Fig. 5, summarized in Table II).

tOmpA folds in liprotides at pH 8–9

tOmpA folding is accompanied by a conspicuous SDS-PAGE band shift, provided the samples are not boiled before loading. Whether the folded protein migrates faster or slower than the unfolded protein can change in response to whether the C-terminal periplasmic domain is included and according to the type of gel used.[28,29] We tested if liprotides could be used to fold tOmpA and used n-dodecyl-β-D-maltoside (DDM) as a control, since tOmpA is known to fold in the presence of DDM micelles.[30,31] tOmpA folds in both liprotide and DDM, but not in OA or aLA alone (Fig. 6).

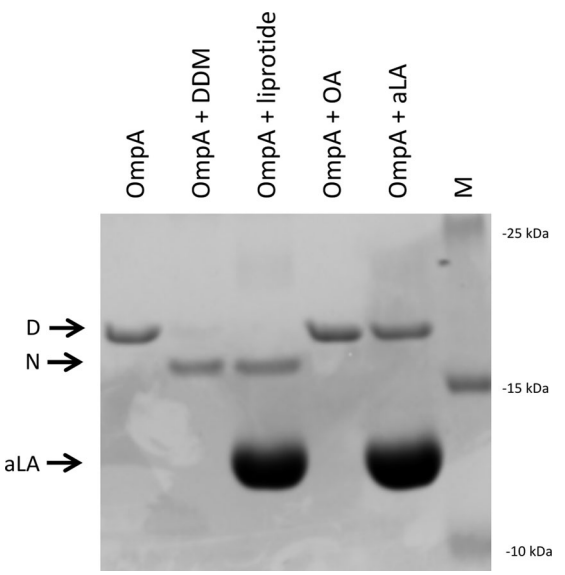

Figure 6. Folding of tOmpA in liprotides followed with SDS-PAGE. SDS-PAGE analysis of the folding of tOmpA under different conditions. Folding is seen as band shift from denatured (D) to native (N). The liprotide dissociates during SDS-PAGE, leading to a strong band of monomeric α-lactalbumin (aLA).

Folding in surfactants such as DDM requires micelles, that is, concentrations above the critical micelle concentration (cmc), which for DDM is 0.17 mM. Liprotides should not face this particular problem since liprotides retain OA even at low concentrations, although some OA may dissociate.[21] To test this, we folded tOmpA over a range of different DDM and liprotide concentrations [Fig. 7(A)]. Folding of tOmpA in both DDM and liprotide was concentration dependent and fitted well to a sigmoidal curve. Data fitting revealed that 50% folding of tOmpA was achieved with 0.23 mM DDM, consistent with a cmc of 0.17 mM. Folding of 2.5, 5, and 10 μM tOmpA was attempted with liprotide and it was found that 0.09, 0.22, and 0.41 mM liprotide (concentrations in units of OA), respectively, were needed for 50% folding of tOmpA. This suggests that the liprotides in the concentrations tested are not limited by a cmc like DDM and other detergents[32] but rather by the ratio between tOmpA and liprotide, which needs to be around 1:40 ([tOmpA]:[OA]).

tOmpA and many other OMPs are known to fold better at high pH, most likely since high pH suppresses aggregation of the OMP in solution. To test this, we investigated folding of tOmpA in DDM and liprotide in buffers with a pH ranging from 6 to 11 [Fig. 7(B)]. tOmpA was able to fold well in DDM at all pH values but had an optimum at pH 11 where all protein was folded. However, folding of tOmpA in liprotides was more pH sensitive and >50% folding was only achieved at pH 8 and 9 where folding was 90% and 100%, respectively. Folding levels were also low at pH 7 and decreased to 12% and 20% at pH 6 and 11, respectively.

In the liprotides, OA is covered by a protein shell which mediates OA's contact with its surroundings. Earlier studies have shown that the liprotides can easily interact with membranes and transfer its OA content.[18,21] To test the effect of the liprotide protein shell on tOmpA folding kinetics, we compared folding rates of tOmpA in liprotide, DDM and small unilamellar vesicles (SUV) of DLPC (Fig. 7). tOmpA was

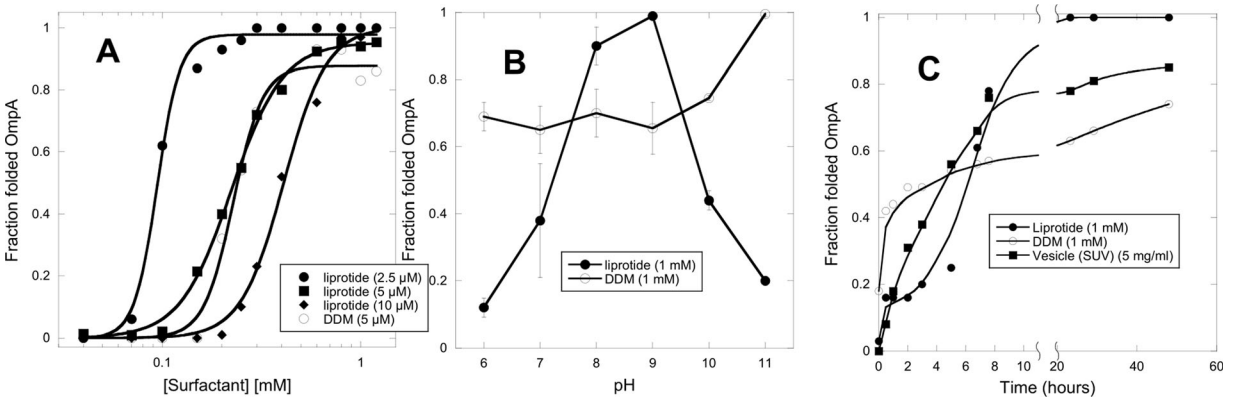

Figure 7. Conditions for folding tOmpA in liprotides. (A) Relative band intensities from SDS-PAGE of folded/unfolded tOmpA (2.5-10 μM) as a function of either DDM or OA in liprotide. (B) Relative band intensities from SDS-PAGE of folded/unfolded tOmpA as a function of pH. (C) Relative band intensities from SDS-PAGE of folded/unfolded tOmpA as a function of incubation time with either liprotide, DDM, or DLPC vesicle.

incubated with its folding partner, samples were taken out at different time points and folding was visualized with SDS-PAGE. Folding in micelles is generally faster than folding in phospholipid,[32] and after 30 min 42%, 16%, and 8% of tOmpA was folded using DDM, liprotide and vesicle, respectively. >50% folding was achieved for tOmpA in DDM and vesicles within 5 h while >50% folding was not seen before 7 h for the liprotide. However, after 1 day, folding had reached close to 100% in liprotides, well above the folding levels of 63% and 78% in DDM and vesicle, respectively.

PagP, tBamA, and OmpF are stabilized by liprotides to a larger degree than by DDM

tOmpA is a robust membrane protein which folds under a wide variety of conditions.[6,7] We therefore turned to more challenging OMPs in the form of PagP, OmpF, and tBamA. Folding of these OMPs are more often incomplete[6] and show slower folding kinetics.[9] However, varying the temperature, buffer conditions and [urea] did not lead to folding of the three OMPs in liprotide except for some conditions that helped PagP fold to 25% (data not shown). Due to these complications, we instead investigated whether the liprotides could stabilize the OMPs before folding in vesicle, effectively working as a chaperone. We did this by first incubating the OMPs with either liprotide, DDM or in buffer, all at low [urea] (0.5 M). At different time points, samples were taken out, mixed with SUVs and incubated for 2 days to allow refolding. SDS-PAGE was again used to follow folding, since band shifts can be used to follow folding of the different OMPs. Complete folding in SUVs was not possible even when the OMPs were directly solubilized in SUVs. Folding levels of $63 \pm 4\%$, $85 \pm 16\%$, and $55 \pm 1\%$ were achieved for PagP, tBamA, and OmpF, respectively (data not shown). Little difference in the folding could be seen when the OMPs were incubated 0–1 h with liprotide, DDM or buffer before incubation with SUVs (Fig. 8). However, when the incubation time was increased to

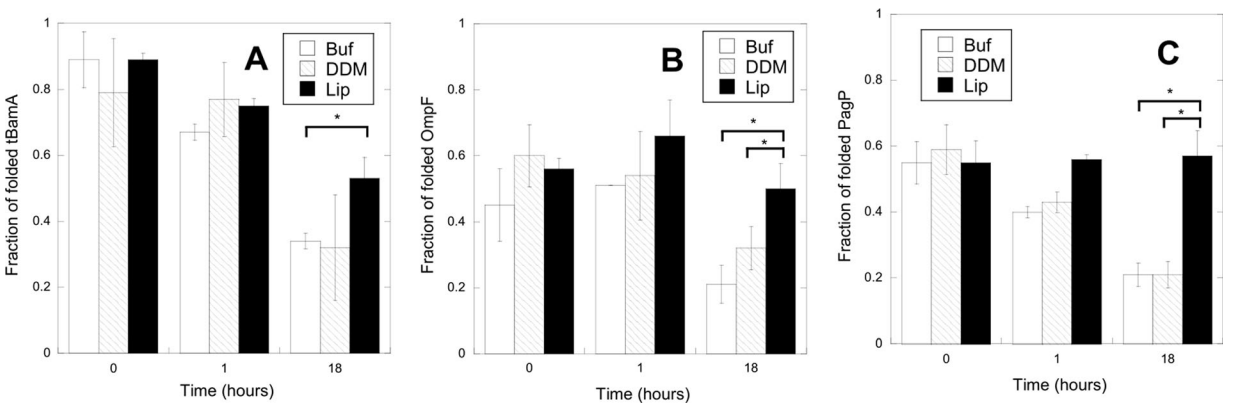

Figure 8. Folding of PagP, OmpF, and tBamA in DLPC vesicles using liprotides to help folding. OMPs were incubated with liprotide, buffer, or DDM and at 0, 1, and 18 h a sample was taken out and mixed with DLPC vesicles. Samples were incubated at 37°C for 2 days and analyzed with SDS-PAGE. Relative band intensities from SDS-PAGE were used to determine folding of (A) tBamA, (B) OmpF, or (C) PagP. Error bars indicate standard deviation of two repeats of the experiment. * designates a significant difference ($^*P < 0.05$).

18 h, there was significantly greater folding of OMPs incubated with liprotides ($57 \pm 8\%$, $53 \pm 6\%$, and $50 \pm 8\%$ for PagP, tBamA, and OmpF, respectively) compared to DDM ($21 \pm 4\%$, $32 \pm 16\%$, and $32 \pm 7\%$) or buffer ($21 \pm 4\%$, $34 \pm 2\%$ and $21 \pm 6\%$) (Fig. 8).

Discussion

We have established that few or no changes are seen for the liprotide structure over the range 0–3 M urea. Folding of OMPs from urea stocks at residual concentrations of 0.25 M urea should therefore not be a problem. It is interesting that aLA in the liprotide retains much of its secondary structure at 6 M urea where apo-aLA shows random coil structure. Far- and near-UV CD experiments on aLA have shown that apo-aLA does not have a clear thermal transition while aLA in liprotide shows a transition that is a bit lower than that of native aLA[33] (35°C and 44°C, respectively). The increased thermal stability and the liprotide stability towards urea suggest that aLA and OA mutually stabilize each other in a specific conformation different from the native structure, thereby making the transition to the completely denatured state unfavorable. The overall core-shell structure of the liprotide also appears to be stable at 6 M urea where a clear core-shell signal is still seen even though the binding of aLA in the shell may have changed. The stability of the liprotide at high pH suggests that folding of tOmpA is not hindered by unfolding of the liprotide at very high pH if the liprotide was also able to stabilize and protect tOmpA from urea.

tOmpA folds in liprotides

We have found that liprotides can be used to fold tOmpA at similar concentrations as DDM. Free OA in solution is not sufficient for tOmpA folding. We interpret this to mean that folding takes place in a hydrophobic OA core stabilized by aLA, and consequently folding with free OA is likely to be hampered by the absence of micelle-like structures; the more complex aggregate phases formed by OA are not suitable for tOmpA incorporation. Similar results have been seen in investigations of OA's antibacterial[16] and anticancer[13] activity, where toxicity is mainly seen when OA is present in the liprotide and not alone. Also in these cases, liprotides must be able to mobilize and present OA in a more accessible fashion than free OA. Earlier studies of a liprotide complex between OA and equine lysozyme[21] showed that bound and free OA were in equilibrium. This likely also applies to other liprotides. Equine lysozyme liprotides still bound OA when diluted to an OA concentration of 28 μM.[21] We find that around 40 OA molecules per tOmpA are needed to achieve 50% folding of tOmpA at 2.5–10 μM tOmpA (100–400 μM OA). Since much of the OA in solution should be bound in the complex at these concentrations, folding is most likely limited by having a too small tOmpA:OA ratio rather than by having too much free OA in solution. Data from SAXS suggest that each liprotide contains around 30 OA molecules, in good agreement with earlier findings.[18] Since around 40 OA molecules per tOmpA is needed to achieve a 50% folding it is likely that folding of tOmpA required more than one liprotide or that one liprotide can provide extra OA to another liprotide to assist in OmpA folding. In the case of folding tOmpA with DDM it has been shown that around 150 DDM molecules assist in folding tOmpA,[31] suggesting that DDM is less effective in folding tOmpA.

Folding of tOmpA in liprotides is highly pH dependent with an optimum at pH 8–9

OmpA generally gives a higher folding yield at high pH.[26] We also saw this for tOmpA folding with DDM, but it is interesting that the liprotides did not follow this trend. At the lower pH of 6 it is likely that folding is poor since the liprotide structure is unstable due to protonation of the OA carboxylate group so that OA starts to precipitate.[17,34] However, over pH range 7–12, while aLA starts unfolding in the absence of OA, the liprotide retains its core-shell structure and liprotide-bound aLA only undergoes small secondary and tertiary structural changes. Nevertheless, little folding of tOmpA occurs in liprotides above pH 9. We attribute this to deprotonation of either the liprotide or tOmpA. For example, deprotonation of Lys residues on aLA will reduce the electrostatic attraction to anionic OA and may also affect interactions with tOmpA. Overall liprotide structure is retained at high pH, implying that it is more favorable for OA to be bound to the aLA shell than to be free in solution, despite the increased pH which helps solubilize OA.[35] It could be that the compaction of the liprotide at higher pH seen with SAXS prevents interaction between OA and OMPs. Nevertheless, the small changes in liprotide structure we do observe may be enough to compromise the folding environment in the OA micelle.

Slow folding of tOmpA using liprotides

Folding of OMPs in the outer membrane of *E. coli* is assisted by different chaperones and lipids. For tOmpA, Skp and lipopolysaccharides play an important role.[36] tOmpA folding yield and kinetics is increased when both Skp and lipopolysaccharide are present, but folding is inhibited when only Skp is present. The role of the lipopolysaccharides might be a general rather than a specific one, since negatively charged lipids can have the same effect.[37] Folding of OMPs with liprotide did not require additional components other than aLA and OA already present in the liprotide, suggesting that binding is not as strong as the case of Skp. The weaker interaction might also

explain the slower folding kinetics of tOmpA in liprotides compared to both micelles and phospholipids.

We have not been able to reconstruct the shape of the tOmpA-liprotide complex by SAXS since purification of the complex using size exclusion chromatography was not possible. The OA core of the liprotide is around 3 nm in diameter, similar to the size of the DDM-tOmpA micelle complex.[31] For obvious steric reasons, part of the aLA on the liprotide surface must be removed to make space for tOmpA. All these rearrangements necessary for tOmpA binding might also rationalize slower tOmpA folding kinetics in liprotides compared to DDM micelles or DLPC vesicles. This also suggests that aLA on the surface of the liprotides might screen and prevent interactions between the OA core and tOmpA. We have also seen in the kinetics data that at the endpoint more tOmpA is folded with liprotides than when using micelles or vesicles, but it is also important to remember that the pH used here was optimized for liprotides and folding with vesicles and micelles would be better at high pH.

Liprotides stabilize and help folding of PagP, tBamA, and OmpF

PagP has a similar size to tOmpA and even though folding with liprotide was poor, some folding could be observed. OmpF and tBamA are around twice as big as tOmpA and PagP and it is likely that the bigger size cause a problem for folding in liprotides. OmpF even folds as a trimer which would produce a structure that is much larger than the liprotide. The liprotides did however prove to be able to interact with the different OMPs to some extent and stabilize them so folding could occur in vesicles at a later time. We also saw that DDM did not work much better than buffer in stabilizing the proteins prior to vesicle insertion, suggesting that it is not simply because we have a hydrophobic environment that the OMPs are able to be stabilized by the liprotides. Instead, the liprotide might be superior to DDM by providing OA to interact with the OMPs while the protein shell can help solubilize the overall structure.

Materials and Methods

Materials

Ca^{2+}-depleted α-lactalbumin from Bovine milk (apo) (aLA, ≥85% pure, L6010) and oleate (OA, ≥95% Pure, O3880) were from Sigma-Aldrich (St. Louis, MO, USA). 1,2-dilauroyl-sn-Glycero-3-phosphocholine (DLPC) was from Avanti polar lipids (Alabaster, AL, USA) and n-Dodecyl-β-D-Maltopyranoside (DDM) was from Anatrace Products (Maine, OH, USA). All other reagents were of high purity or HPLC grade.

Expression and purification

Plasmids (pet22b) of either tOmpA (transmembrane OmpA, residues 1–176 of the N-terminal domain without leader sequence), PagP[6] (residues 26–186), tBamA[9] (transmembrane BamA, residues 425–810) or OmpF[9] (residues 23–362) were transformed into *E. coli* BL21(DE3) cells by electroporation. Cells were incubated in LB media at 37°C and expression was induced by addition of 1 mM IPTG at $OD_{600} = 0.8$. The cells were incubated for 4 h before being harvested by centrifugation at 4000*g* for 20 min. Cells were resuspended in 50 mM Tris pH 8 containing Roche complete ultra-tablet (protease inhibitor). Inclusion bodies were released by sonication for 5 min with an on/off cycle of 10 s at 20% power using a Q500 Sonicator (Qsonica, CT, USA) in an ice water bath. Inclusion bodies (IBs) were collected by centrifugation at 5000*g* for 15 min and resuspended in 1% Triton X-100, 50 mM Tris pH 7.5 using a glass homogenizer and subsequent shaking at 37°C for 1 h. IBs were harvested by centrifugation at 5000*g* for 15 min and washed two times in 50 mM Tris pH 8, 2 mM EDTA. After the last wash, IBS were dissolved in 8 M urea, 10 mM Tris pH 9, and debris was removed by centrifugation at 30,000*g* for 30 min. Protein purity was assessed by SDS-PAGE, showing >95% purity. Protein concentration was determined using absorbance at 280 nm with extinction coefficients ($E_{1\%}$) of 25.0 (tOmpA), 43.1 (PagP), 23.4 (tBamA), and 14.6 (OmpF).

SDS-PAGE

SDS-PAGE was used to monitor the purity of purified proteins and to check folding of OMPs. Protein was loaded with a reducing buffer at 0.1 mg/mL. tOmpA was run in 15% BisTris SDS-PAGE gels while PagP, tBamA, and OmpF were run in 4–15% Laemmeli SDS-PAGE gels to resolve folded from unfolded bands. Samples were not heated before loading on SDS-PAGE except for control samples of unfolded OMPs, which were heated to 95°C for 5 min. Densitometry quantification of bands was performed using ImageJ.[38] All gels were stained with Coomassie Brilliant Blue.

Liprotide formation

aLA was dissolved in MilliQ water to ~10 mg/mL and OA was dissolved in MilliQ and 20% ethanol to 125 mM. Liprotides were made by mixing 6 mg/mL aLA with 6.3 mM OA in 10 mM glycine pH 8.5 and heating it to 80°C for 15 min.[17,18] 8 M urea, 10 mM glycine pH 8.5 was used for samples containing urea. To change the pH of the solutions, either 50 mM $NaHPO_4$ (pH 6, 7, 7.4, and 8) or 50 mM glycine (pH 8.5, 9, 10, 11, and 12) was used. The final pH solutions had 1.7 mM glycine with either 50 mM $NaHPO_4$ or 50 mM glycine. pH was measured with

a PHM210 pH meter (Radiometer Analytical, Villeurbanne, France) and adjusted using either NaOH or HCl.

Folding of OMPs

OMPs were folded by diluting a 3.2 mg/mL protein solution containing 8 M urea to a 0.1 mg/mL protein solution containing 0.25 M urea, 10 mM glycine pH 8.5 and either DDM, liprotide, or DLPC vesicles. tOmpA was incubated in either 50 mM $NaHPO_4$ (pH 6, 7, and 8) or 50 mM glycine (pH 8.5, 9, 10, and 11) with different concentrations of DDM or liprotide. Samples were incubated at 23°C and folding was followed with SDS-PAGE.

Folding in DLPC vesicles

20 mg/mL DLPC were dissolved in 10 mM glycine pH 8.5. Vesicles were created by sonication for 10 min with an on/off cycle of 10 s at 20% power using a Q500 Sonicator (Qsonica, CT, USA) in an ice water bath. The vesicles were stored at 4°C and used within 1 day. 0.2 mg/mL OMP was incubated at 37°C with either 1 mg/mL liprotide, 1 mM DDM or in buffer solution of 10 mM glycine pH 8.5. At 0, 1, and 18 h, 50 μL samples was taken out and mixed with 50 μL of 20 mg/mL DLPC. Samples were then incubated at 37°C for 2 days and folding of OMPs was followed with SDS-PAGE. The experiments were repeated twice.

Folding kinetics

Folding of tOmpA over time was followed by incubating 0.1 mg/mL tOmpA with 1 mg/mL liprotide, 1 mM DDM, or 5 mg/mL DLPC vesicles at 23°C. Samples were taken at different time points and folding was quenched by incubation in SDS-PAGE loading buffer and followed by SDS-PAGE.

Trp fluorescence

Spectra were recorded with an LS-55 luminescence spectrometer (Perkin Elmer, Waltham, MA) at a protein concentration of 0.2 mg/mL. Measurements were done by varying either [urea] in 10 mM glycine pH 8.5 or by varying the pH using 50 mM $NaHPO_4$ (pH 7.4 and 8) or 50 mM glycine (pH 9, 10, 11, and 12). Measurements were recorded in a 10 mm quartz crystal cuvette with excitation at 280 nm and emission at 300–450 nm. Measurements were run at 23°C with a scan speed of 200 nm/min and an accumulation of three scans per spectrum.

CD: Spectra were recorded with a Jasco-810 spectropolarimeter (Jasco, Hachioji, Japan) at a protein concentration of 1 mg/mL. Measurements were done by varying either [urea] in 10 mM glycine pH 8.5 or by varying the pH using 50 mM $NaHPO_4$ (pH 7.4 and 8) or 50 mM glycine (pH 9, 10, 11, and 12). Measurements were recorded in a 0.1 mm quartz crystal cuvette at 190–250 nm and data cut when the HT voltage was above 500. Measurements were run at 23°C with a scan speed of 100 nm/min and an accumulation of five scans per spectrum.

Small-angle X-ray scattering (SAXS)

Eighty microliters sample of 2 mg/mL protein was loaded on a flux- and background optimized NanoSTAR SAXS camera at Aarhus University (Bruker AXS).[39] Measurements were performed at 20°C with an acquisition time of 10 min at a wavelength of $\lambda = 1.54$ Å and all data is expressed by the scattering vector given by $q = 4\pi \sin(\theta)/\lambda$ where 2θ is the scattering angle. Solutions containing 0–6 M urea, 10 mM glycine pH 8.5 or 50 mM $NaHPO_4$ (pH 7.4 and 8) or 50 mM glycine (pH 9, 10, and 11) were used as background. Water was used as a calibration standard. Background subtraction as well as conversion to absolute scale was done using the SUPERSAXS program package (C. L. P. Oliveira and J. S. Pedersen, unpublished).

The $p(r)$ function was determined using indirect Fourier transformation[40] to get model independent information on the real space distribution of distances in the sample and thereby insight in the shape and size of the particles. The protein–lipid complexes were modeled as a spherical core-shell model with the core consisting of the lipid and the shell consisting of flexible protein.[12] Electron densities of the background were calculated for the buffers with mixtures of urea and water, so that they could be used for calculating the excess electron densities in the respective solutions with urea and water. Model dependent parameters were extracted by fitting the models to experimental data.

SUPPLEMENTARY MATERIAL is available at <link> and contains fits to SAXS spectra of liprotides in 0–6 M urea and SDS-PAGE analysis of OMP folding into DLPC vesicles from different starting conditions.

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Disclosure

The authors have no conflict of interest to declare.

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